Protocol: Extract lens protein, Prevention, Treatment

2016年10月18日　　下午 02:01

Materials:

- Tris buffer
  - Trizma® base (Primary Standard and Buffer, ≥99.9% (titration), crystalline)
  - DL-Dithiothreitol (≥98% (TLC), ≥99.0% (titration))
  - EDGMA Ethylenediaminetetraacetic acid
- Priacanthus macracanthus (from fish market)
- Prevention
  - L-Glutathione oxidized (≥98% (HPLC))
  - L-Glutathione reduced (≥98.0%)
- Treatment
  - 25-hydroxycholesterol (≥98%)

Procedure:
Setting up a Cataracts Model

- **Pre-lab: Find an appropriate wavelength to measure absorbances**
- Prepare 2 groups of fish nuclear protein (in dh2o)
  - Control (no treatment)
  - + 200uL of 35% h2o2
- Let sit for 10 min
- Measure Abs with SpectroVis
  - Blank with water
  - Get full spectrum
- Results: Highest absorbance at 397.5 nm
-

- **Day 1:**
- Dissect fish (紅目鰱) eyes, extract both lenses (nucleus)
  - Rinse/clean gently with 2 mL Tris buffer
- Place each sample in 12~16 mL Tris buffer (15mL centrifuge tube)
- Tape onto benchtop rotator and gently shake overnight at 70rpm

- **Day 2:**
- Transfer solution to __mL centrifuge tubes (15mL centrifuge tubes); discard lenses
- Centrifuge at 4,500 rpm for 10 min (to pellet insoluble proteins)
  - Transfer supernatant to new 15 mL tube
- Prepare tubes with H2O2, Tris, and protein solution
-

3/22/2016 Initial Protocol

Thursday, March 24, 2016 4:41 PM

Goal: figure out which solution the lens dissolves in best (in order to measure opacity)

First method:
Procedure:

- ddh2o lens
- Put lenses in eppendorf with 7ml water
  - Vortex for 6 min
- Next time try ddh2o lens in ethanol and methanol

Modified method:
Goal: Extract soluble proteins from fish outer and nuclear lens

Protocol (CREDS TO THE WANG AND ONLY) *can be done with or without the gooey part

- Extract both lens out of the fish eye
- Rinse both lens with saline solution
- Put each lens into a culture tube filled with 5mL ddH2O
- Settle on shaker and gently shake for 20hr
- Centrifuge both samples at 30000 Rpm for 6 mins
- Extract 3mL of supernatant (not including the insoluble protein) from each sample into a cuvette
- Do not discard culture tubes
- Measure OD for initial value
- Add 3mL ddH2O to culture tube
- Repeat steps 4-6

3/24 SDS PAGE

Thursday, April 7, 2016 1:47 PM

> supernatant containing solubilized proteins >> run sds-page
[load 2 to 50ug total per well]

** we have ~10mg/ml = 10ug/ul [need to +4x laemmli SB +Bme]
>> 10ul sample : ~3.5ul SB+Bme

>> samples:
10ul of [5ul sample+5ul water >> 50ug total]
10ul of [2.5ul sample+7.5ul water] >> 25ug total
10ul of [1ul sample+9ul water] >> 10ug total

1. protein ladder
2. 1x SB
3. 10ug cortex
4. 25ug cortex
5. 50ug cortex
6. 1x SB
7. 10ug nucleus
8. 25ug nucleus
9. 50ug nucleus
10. 1x SB

Coommassie blue to stain proteins, wash overnight with dH2O

4/15 Cataracts model trial 1: H2O

Monday, October 17, 2016 4:00 PM

Wavelength: 395nm
Absorbance: increase: more opaque; potentially more protein
Goal: test the change in absorbance over time of the protein solution solubilized in water

Percent Change from initial:
Concentration: H2O2

21h	Control (-)	0.1 mM	0.3 mM	0.5 mM
Pig nucleus	37.1	-5.7	10.4	18.3
Pig cortex	38.1	23.2	26.8	19.9
Fish nucleus	34.5	28.5	14.1	10.4
Fish cortex	33.5	23.2	5.8	12.3
21h after cen	Control (-)	0.1 mM	0.3 mM	0.5 mM
Pig nucleus	29.1	-9.9	10.5	9.2
Pig cortex	19.2	17.4	5.9	27.5
Fish nucleus	23.4	13.0	14.4	6.1
Fish cortex	20.0	-8.0	19.0	8.9

70h	Control (-)	0.1 mM	0.3 mM	0.5 mM
Pig nucleus	20.0	-7.0	8.4	16.9
Pig cortex	-15.6	26.4	-23.9	-21.8
Fish nucleus	10.8	8.4	26.3	4.8
Fish cortex	18.5	-19.7	3.5	5.3
70 h after cen	Control (-)	0.1 mM	0.3 mM	0.5 mM
Pig nucleus	20.0	-7.0	8.4	16.9
Pig cortex	-15.6	26.4	-23.9	-21.8
Fish nucleus	10.8	8.4	26.3	4.8
Fish cortex	18.5	-19.7	3.5	5.3

90h	Control (-)	0.1 mM	0.3 mM	0.5 mM
Pig nucleus	38.6	11.0	11.4	33.3
Pig cortex	50.5	42.1	17.1	30.2
Fish nucleus	30.7	57.2	57.7	10.9
Fish cortex	36.5	29.5	54.1	7.7
90h	Control (-)	0.1 mM	0.3 mM	0.5 mM

Pig nucleus	34.0	-8.0	10.5	25.7
Pig cortex	15.7	21.4	8.3	1.5
Fish nucleus	12.6	15.4	34.9	5.6
Fish cortex	18.2	28.7	45.4	5.3

4/21 Cataracts model trial 2: Saline

Monday, October 17, 2016 4:32 PM

Goal: test the change in absorbance over time of the protein solution solubilized in saline
After c= absorbance value measured after centrifuging the protein solution (to see how much protein is lost)

PERCENT CHANGE from initial

20h	Control (-)	0.1 mM	0.5 mM	1 mM
Nucleus	204	46	390	523
Cortex	216	352	378	255
20h after c	Control (-)	0.1 mM	0.5 mM	1 mM
Nucleus	15.9	-25.4	254	233
Cortex	4.55	-15.9	-20.0	-53.4

42h after c	Control (-)	0.1 mM	0.5 mM	1 mM
Nucleus	258	61.2	1858	1149
Cortex	432	155	107	133
42h after c	Control (-)	0.1 mM	0.5 mM	1 mM
Nucleus	-10.1	-83.6	-83.6	-85.1
Cortex	-15.9	-2.27	12.7	-27.6

70h	Control (-)	0.1 mM	0.5 mM	1 mM
Nucleus	351	176	445	147
Cortex	655	161	158	147
70h after c	Control (-)	0.1 mM	0.5 mM	1 mM
Nucleus	-88.3	80.6	-53.7	-62.1
Cortex	129.5	-34.1	-12.7	-29.3

94h	Control (-)	0.1 mM	0.5 mM	1 mM
Nucleus	397	318	306	69.0
Cortex	848	161	173	157
94h after c	Control (-)	0.1 mM	0.5 mM	1 mM
Nucleus	-8.70	207	-29.9	-47.1
Cortex	264	-50.0	-5.45	831

4/29 Cataracts model trial 3: Saline

Monday, October 17, 2016 4:38 PM

Goal: replicate previous trial

PERCENT CHANGE from intial				
Nucleus				
Time (hr)	Control (-)	0.1 mM	0.5 mM	1 mM
0	0	0	0	0
19	72	232	58	25
48	193	239	181	81
72	270	307	258	117
Cortex				
Time (hr)	Control (-)	0.1 mM	0.5 mM	1 mM
0	0	0	0	0
19	195	160	117	71
48	493	431	355	245
72	627	600	461	338

5/11 Cataracts model trial 4: Phosphate buffer saline

Monday, October 17, 2016 4:42 PM

Goal: test the change in absorbance over time of the protein solution solubilized in PBS and Tris

PHOSPHATE BUFFER SALINE (PBS)

PERCENT CHANGE from initial				
20h	Control (-)	0.1 mM	.5 mM	1 mM
Nucleus	319.1	320.8	21.3	21.2
44h	Control (-)	0.1 mM	.5 mM	1 mM
Nucleus	516.0	597.2	59.6	369.2
116h	Control (-)	0.1 mM	.5 mM	1 mM
Nucleus	3091.5	1834.7	344.7	613.5

Note: the proteins turned green?!

TRIS BUFFER

PERCENT CHANGE from initial				
20h	Control (-)	0.1 mM	.5 mM	1 mM
Nucleus	-25.81	-30.00	32.35	98.89
44h	Control (-)	0.1 mM	.5 mM	1 mM
Nucleus	-74.19	-70.00	66.18	435.56
116h	Control (-)	0.1 mM	.5 mM	1 mM
Nucleus	35.48	163.33	276.47	3233.33

5/19 Cataracts model trial 5: TRIS final trial

Monday, October 17, 2016 4:51 PM

Goal: confirm that we can use tris as our buffer to solubilize fish lens proteins

PERCENT CHANGE OVER TIME

Tris 1							Tris 2			
	0h	15h	20h					0h	15h	20h
control	0.000	0.015	0.007				control	0.000	0.016	0.001
0.1mM	0.000	-0.009	-0.019				0.1mM	0.000	0.001	-0.003
1mM	0.000	0.009	0.002				1mM	0.000	0.022	0.019
10mM	0.000	0.027	0.031				10mM	0.000	0.022	0.026

5/25 & 6/4 Prevention trials

Monday, October 17, 2016 4:56 PM

Goal: ADD GSH AND GSSG (positive control) into our (TRIS) cataracts model

Prediction: GSH would prevent H2O2 from denaturing the fish lens protein, GSSG is supposed to be our positive control

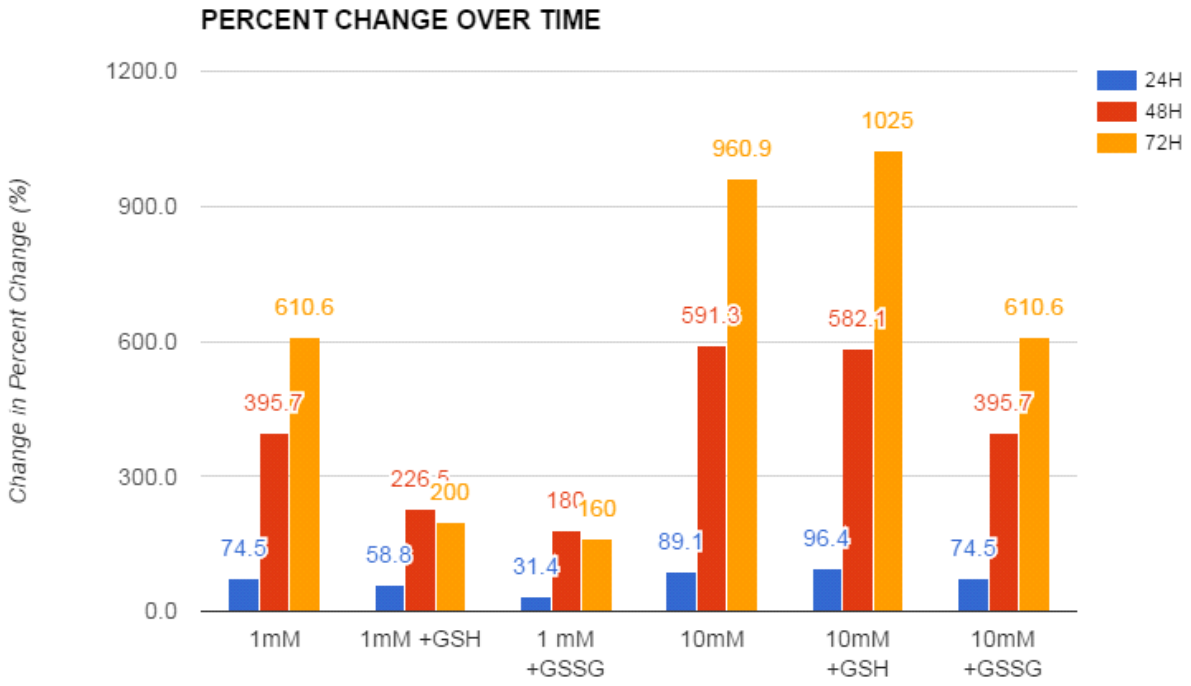
Observations:

- we added half of GSSG bc GSSG breaks into two GSH (<this is wrong)
- 5/25
  - Added 0.4mg of GSH and GSSG... that was basically impossible to measure, very likely to have many human errors
- 6/4
  - Added 4mg of GSH and 2mg of GSSG

Results:

5/25

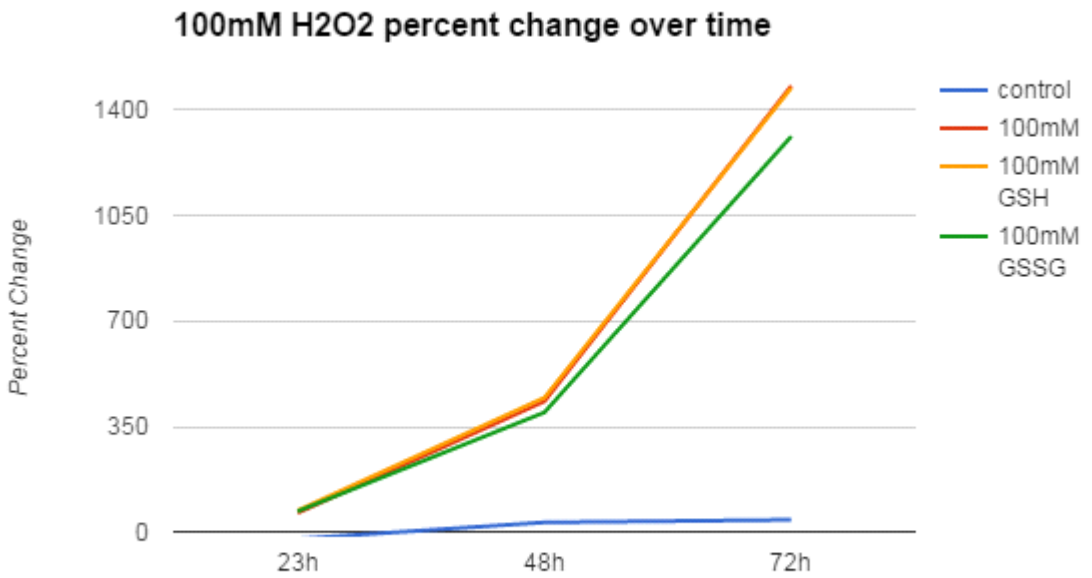
PERCENT CHANGE from initial			
Time (hr)	24H	48H	72H
1mM	74.5	395.7	610.6
1mM +GSH	58.8	226.5	200
1 mM +GSSG	31.4	180	160
10mM	89.1	591.3	960.9
10mM +GSH	96.4	582.1	1025
10mM +GSSG	74.5	395.7	610.6



6/4

PERCENT CHANGE IN ABSORBANCE

	23h	48h	72h
control	-20.0	34.3	42.9
100mM	65.2	434.8	1478.3
100mM GSH	73.5	446.9	1471.4
100mM GSSG	70.9	398.2	1310.9



Analysis:
5/25 trial
- GSSG was actually more effective (less change in % change), not really a positive control...
- both 10mM GSSG and 100mM GSSG have the least change in % change, and we thought it made sense because if we added the same amount of GSSG as GSH, and GSSG breaks into 2 GSh, we would have double the amount of GSH in the GSSG tube

6/4 trial
- GSSG is still more effective (less change in % change) than GSH, but compared to last trial, the difference in percent change between GSSG and GSH is less this time. (this is probably bc of the halved amount of GSSG)
- 10mM GSH actually not as effective as GSSG nor just h2o2

thoughts...
- this is why we dont add GSH.. not as effective as converting GSSG back to GSH (letting the cycle flow)
- GSH to GSSG is endothermic?!??!?! or the other way around
- GSH requires collision? more difficult than dissociation (also, GSSG-->GSH is faster)

6/26 Prevention trial 2 & 3

2016年10月17日　　下午 05:09

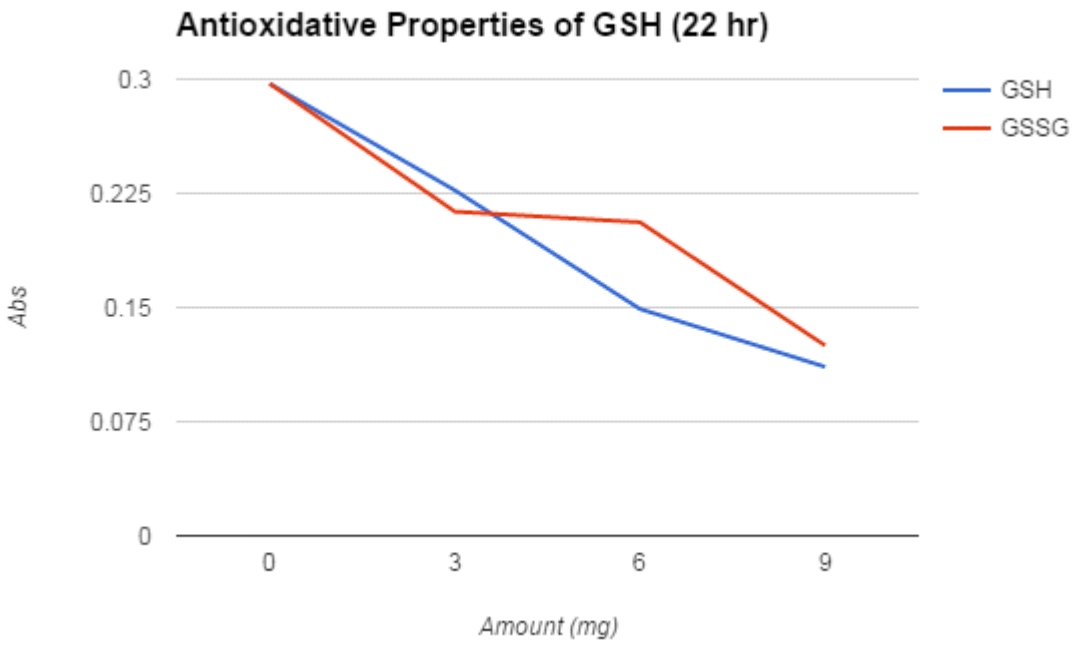
Goal: using a slightly altered protocol to test the percent change in absorbance of protein solution

Protocol:

1. cut nucleus into small pieces
2. vortex
3. centrifuge 10 min
4. remove super
5. 6 samples + 1 control 3mL each
6. add GSH/GSSG
7. add 3.89 uL 35% H2O2 = 10mM

Percent change in absorbance after 22h

(mg)	GSH	GSSG
0	0.297	0.297
3	0.227	0.213
6	0.149	0.206
9	0.111	0.125


Antioxidative Properties of GSH (22 hr)
0.3
0.225
0.15
0.075
0
Abs
0
3
6
9
Amount (mg)
GSH
GSSG

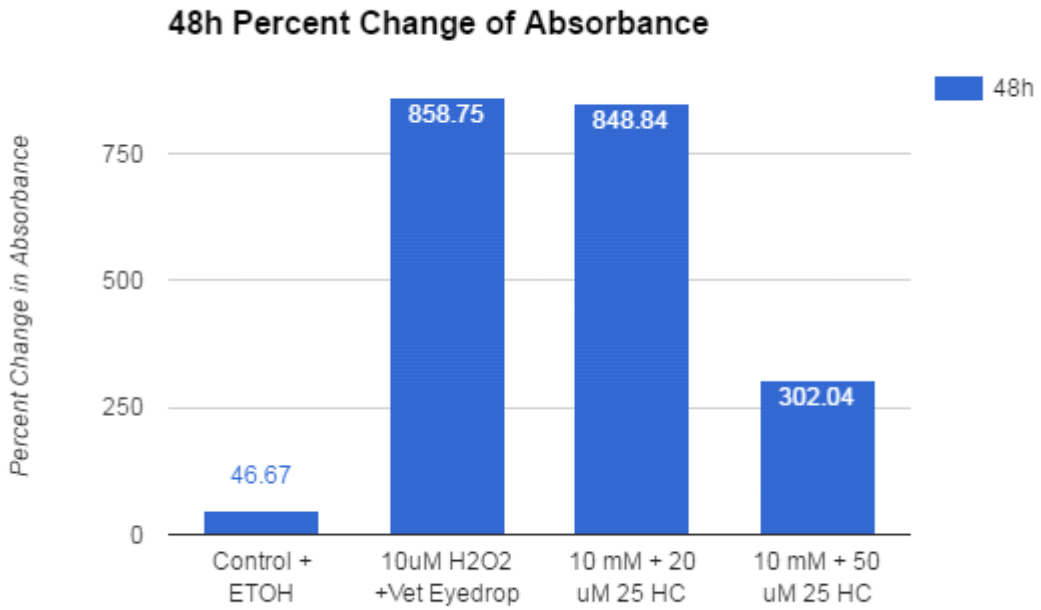
6/14 Treatment trial 1

2016年10月17日　　下午 05:17

Goal:

Percent change in absorbance over time

	48h
Control + ETOH	46.67
10uM H2O2 +Vet Eyedrop	858.75
10 mM + 20 uM 25 HC	848.84
10 mM + 50 uM 25 HC	302.04

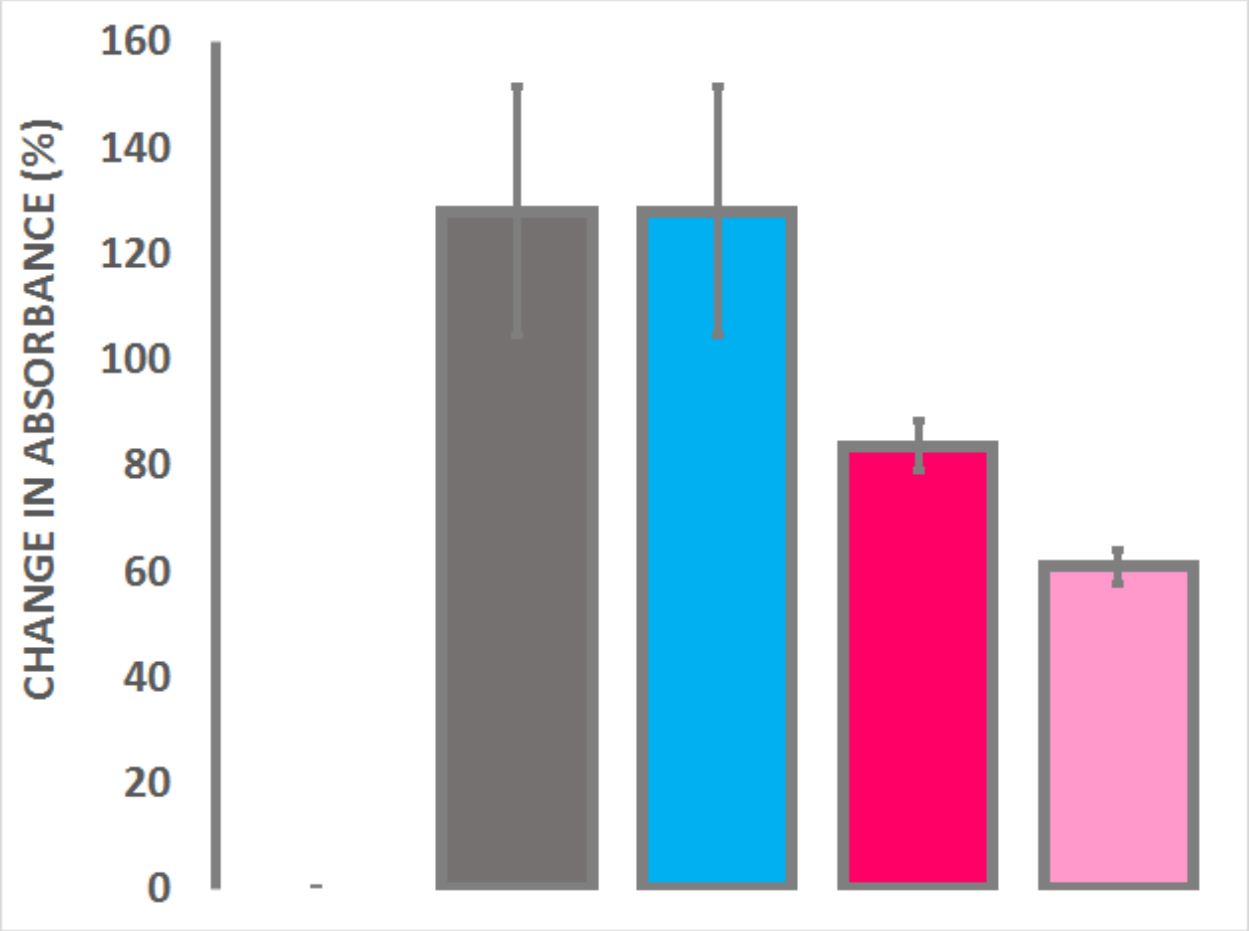


9/19 Treatment trial 2

2016年10月17日　　下午 05:22

REPLICATION OF 6/14 TRIAL

	PERCENT CHANGE from initial			
	o: add h2o2	24h (first added treatment)	48h	72h
	0	68.1	104.4	130.8
	0	95.2	151.6	275.8
control average	0	81.6	128	203.3
	0	19.1	33.4	102.6
	0	13.5	23.6	72.5
		47.7	79.3	98.3
		50	78.4	116
		58.5	93.1	156.2
20um average	0	52.1	83.6	123.5
	0	5.7	8.2	29.6
	0	3.3	4.8	17.1
		41.5	64.2	125.4
		36.1	54.5	80.5
		40.4	64	49.1
50um average	0	39.3	60.9	85
	0	2.8	5.6	38.3
	0	1.6	3.2	22.1
vet 1 drop		51	64.9	
vet 3 drops		6.1	120.4	
average	0	81.6	128	
stdev	0	19.1	33.4	
sem	0	13.5	23.6	



h2o2 to plateau, then add 25hc			
23-Sep			
	Initial (Time 0)	24h	48h
Control 1	0	0	-2.2
Control 2	0	-1.2	-3.6
CONTROL	0	-0.6	-2.9
stdev	0	0.860227228	1.028496066
sem	0	0.608272506	0.727256543
20uM 1	0	-1.6	-4.9
20uM 2	0	-12.1	-14.1
20 uM	0	-6.9	-9.5
stdev	0	7.366907748	6.483640959
sem	0	5.209190425	4.584626489
50uM 1	0	-1.3	-4.2
50uM 2 (more diluted)	0	-2.3	-6.5
50 uM	0	-1.8	-5.3

stdev	0	0.716040757	1.561341601
sem	0	0.506317275	1.104035234